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DARPA ANTIBODY TECHNOLOGY PROGRAM

STANDARDIZED TEST BED FOR ANTIBODY CHARACTERIZATION: CHARACTERIZATION OF AN MS2 SDAB PRODUCED BY U.S. NAVAL RESEARCH LABORATORY

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14. ABSTRACT:

The selection of antibodies for inclusion in a final assay format has primarily relied on an antibody's performance in an enzyme-linked immunosorbent assay, with little regard for quantification of the full spectrum of variables that affect antibody–antigen interactions. The Joint Product Management Office for Biosurveillance (JPMO BSV), Critical Reagents Program instituted a quality program for the standardization of test methods to support more-comprehensive characterization and comparison of the physical and functional properties of antibody reagents within its repository. The development and standardization of antibody testing provides JPMO BSV with an invaluable platform for providing consistent, high-quality assays and reagents for current biodetection platforms and for developing and validating future systems. This platform will be used to characterize and evaluate the MS2 recombinant antibody produced and supplied by the Defense Advanced Research Projects Agency (DARPA; Arlington, VA)-funded U.S. Naval Research Laboratory (Bethesda, MD) for affinity and stability enhancements. The study provides standardized parametric data on antibody properties and performance and contributes to the development of a decisional analysis tool used to expand the confidence level for selection of antibody-based reagents that will optimize field operational and performance metrics for future detection and diagnostic platforms.

15. SUBJECT TERMS

Antibody	Biosurveillance	Antibody Technology Program (ATP)
Quality	MS2 coat protein (MS2CP)	Enzyme-linked immunosorbent assay (ELISA)

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PREFACE

The work described in this report was supported by Defense Advanced Research Projects Agency funding. The work was started in October 2010 and completed in September 2012.

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1. INTRODUCTION

Current platforms for the detection and diagnosis of biothreat agent exposure depend on the use of antibodies to recognize and bind to specific antigens. To date, the selection of antibodies for inclusion in a final assay format has primarily relied on an antibody's performance in an enzyme-linked immunosorbent assay (ELISA), with little regard for quantification of the full spectrum of variables that affect antibody–antigen interactions. The Joint Product Management Office for Biosurveillance (JPMO BSV), Critical Reagents Program (CRP) instituted a quality program for the standardization of test methods to more fully characterize and compare the physical and functional properties of antibody reagents in its repository. The development and standardization of antibody testing provides the JPMO BSV with an invaluable platform for providing consistent, high-quality assays and reagents for the existing biodetection platforms and for developing and validating future systems. This platform will be used to characterize the MS2 single domain antibody (sdAb), which was produced at the U.S. Naval Research Laboratory (NRL; Bethesda, MD) for the Defense Advanced Research Projects Agency (DARPA; Arlington, VA) Antibody Technology Program (ATP).

The DARPA ATP focuses on developing technologies to enhance the thermal stability and binding affinity of a given antibody. Functioning as an independent testing laboratory for this program, the U.S. Army Edgewood Chemical Biological Center (ECBC; Aberdeen Proving Ground, MD) provided specific technical support on immune reagents and defined the government-supplied, antibody–antigen pairs. The goal of this project was two-fold: (a) select, develop, and standardize the methods for characterizing the de novo thermal and binding properties of select reagents to be used by DARPA-funded investigators, and (b) use those methods to validate the changes in antibody thermal stability and binding affinity that were achieved by the DARPA investigators. The antibody chosen for this project was the MS2 recombinant single-chain fragment (scFv) antibody produced at ECBC (*1*), which detects an MS2 coat protein (MS2CP) that forms the capsid for the MS2 bacteriophage. The focus of the work described herein was to evaluate the MS2 antibodies supplied by the DARPA-funded investigator, NRL, for affinity and stability enhancements. The results of this study provide standardized parametric data on antibody properties and performance. This information will also contribute to the development of a decisional analysis tool to expand the confidence levels for the selection of antibody-based reagents that will optimize the field operational and performance metrics for future detection and diagnostic platforms.

2. MATERIALS AND METHODS

2.1 MS2 scFv and MS2CP

The original MS2 scFv antibody was produced from a plasmid that was supplied by Ellen Goldman at NRL. The plasmid was designated as Gv1, and the sequence was cloned into a pET-22b(+) plasmid (EMD Millipore; Billerica, MA). The protein was produced and eluted in 20 mM sodium phosphate (pH 8.0), 0.5 M sodium chloride, and 0.5 M imidazole. Peak fractions were then collected and separated on a 16/60 Superdex 200 gel filtration column (GE Healthcare Life Sciences; Pittsburgh, PA), and fractions that corresponded with a monomeric protein were collected and flash-frozen in liquid nitrogen. These fractions were then provided (along with sequence data) to NRL as baseline material for modification.

The MS2CP was produced from a pET-28a(+) plasmid (Novagen, Inc.; Madison, WI) with the MS2CP sequence inserted with an amino acid substitution of an arginine at position 83 construct engineered by DNA2.0, Inc. (Menlo Park, CA; www.DNA20.com). MS2CP was produced and eluted with 300 mM imidazole in pH 7.4 phosphate-buffered saline (PBS; Sigma-Aldrich Company LLC; St. Louis, MO). Peak fractions were collected, and buffer was exchanged into PBS (pH 7.4) using 470 mL of packed volume of Sephadex G-25 (Amersham Biosciences Corporation; Piscataway, NJ) fine gel chromatography media and provided to NRL as an antigen for the MS2 antibody.

2.2 Ultraviolet-Visible (UV-Vis) Spectrophotometry

A NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.; Waltham, MA) was used to determine the MS2 sdAb concentrations and the absorbance of light at 280 nm (A_{280}) for the samples supplied by NRL. The A_{280} value is influenced by the number of tryptophan and tyrosine residues in a given protein. For this reason, the extinction coefficient is used in conjunction with the A_{280} value to determine an accurate concentration. The MS2 sdAb concentrations were determined by dividing the average A_{280} value by 1.44, which is the extinction coefficient for an sdAb. Each reading required a 2 µL sample, which was placed on the sample pedestal. The arm of the instrument was lowered, creating a liquid column between the top of the arm and the surface of the pedestal; this was the path length through which the laser passed. The instrument was blanked using PBS, and readings were taken in triplicate. A positive control, bovine γ-globulin (BGG; Bio-Rad Laboratories; Hercules, CA), was also tested to validate the instrument operation.

2.3 Electrophoresis

Molecular weight and purity data were collected using an Experion automated electrophoresis system (Bio-Rad). The Experion system employs microfluidic technology to automate electrophoresis for protein analysis. The microfluidic chip, in conjunction with the Experion reagents, electrophoresis station, and software, are designed to accomplish separation, staining, destaining, detection, and basic data analysis. The Experion Pro260 analysis kit uses engineered lower and upper internal alignment markers to provide clean baselines, accurate molecular weight sizing, and quantitative protein analysis (*2*). The Pro260 analytical software

also determines sample purity by calculating the percent mass of the separated proteins in a sample. For Experion analysis, each of the NRL MS2 sdAbs was standardized to a final concentration of 1 mg/mL by diluting it in PBS. The BGG control and the NRL samples were then processed using a validated procedure specified in the Bio-Rad Experion Pro260 analysis kit, rev. C (*3*). Briefly, a Pro260 microfluidic chip was prepared by adding 12 µL of Pro260 gel and gel stain to the designated wells. The chip was then placed on the priming station and primed for 1 min at the medium (B) pressure setting. Priming filled the fluidic channels with gel, which was used by the instrument to form a barrier between samples during the run. The sample was reduced with dithiothrietol (Sigma-Aldrich) and denatured in the kit-provided sample buffer at 95 °C before it was applied to the primed chip. The chip was then placed in the instrument, and the lid was closed, which lowered the sample needles into the wells. The instrument was operated using the Experion software, and each chip took 30 min to complete. All samples were run in triplicate alongside one sample of the BGG control and the Pro260 ladder. All analyses were performed using the Experion software.

2.4 Dynamic Light Scattering (DLS)

DLS was used to paint a picture of how the proteins behaved in solution. DLS data indicate whether a protein is in solution by measuring the polydispersity, hydrodynamic radius, and molecular weight of the sample. Prediction algorithms within the DLS software produce a range of values for the protein under evaluation. For DLS analysis, five 20 µL aliquots of the NRL MS2 sdAbs, along with the control bovine serum albumin (Sigma-Aldrich), were placed into a quartz 384-well plate (Wyatt Technology Corporation; Santa Barbara, CA) and centrifuged for 2 min at 239 × *g* to remove trapped air bubbles from the samples. Mineral oil (Sigma-Aldrich) was applied to the top of each sample to prevent sample evaporation. The plate was then placed into a DynaPro temperature-controlled plate reader (Wyatt Technology). Each well was scanned 10 times for 5 s each at 25 °C. Values were averaged to provide measurements of polydispersity, hydrodynamic radius, percent mass, and molecular weight for each sample using the Dynamics software (Wyatt Technology). The results of three wells were averaged and reported.

2.5 Differential Scanning Calorimetry (DSC)

DSC was used to obtain a quantitative melting temperature (T_m) for each of the NRL MS2 sdAb proteins. The T_m should predict the results of subsequent ELISA and surface plasmon resonance (SPR) thermostability testing. A T_m above 70 °C predicts that the percentage of antibody activity after the thermal stress test will remain above 50%. A T_m below 70 °C predicts at least a 50% decrease in antibody activity after the thermal stress test. For DSC experiments, samples were diluted to 0.5 mg/mL and dialyzed overnight in PBS (pH 7.4). Before analysis, samples were degassed for 5 min and then injected into the sample cell of a VP-DSC microcalorimeter (MicroCal of Malvern Instruments; Malvern, UK). Dialysis buffer was added to the reference cell of the calorimeter, and a buffer scan was used as the baseline for all experiments. The duplicate samples were each scanned from 15 to 100 °C at a rate of 60 °C/h. The transition midpoint of the protein was determined by data analysis using Origin 7.0 software (MicroCal).

2.6 Thermal Stress Test

Before applying heat, all samples were diluted to 1 mg/mL to negate any protective effects that may have been due to concentration (*2*). The NRL thermally stabilized antibody (2cams2) was diluted to 1 mg/mL in 1× PBS and divided into five tubes. One aliquot was kept on ice for the duration of the experiment and was marked "time 0". The remaining four aliquots were heated to 70 °C on a calibrated heat block for 15, 30, 45, and 60 min each. After each time point, the corresponding aliquot was removed and placed in an ice bath. These samples were then tested for activity.

2.7 ELISA

ELISAs were performed in triplicate using standard techniques. After the thermal stress test, each sample was diluted to 1 µg/mL in PBS and used to coat one row each of three Nunc MaxiSorp 96-well plates (Thermo Fisher Scientific). The plates were incubated at 4 °C overnight. In the morning, each plate was washed in 1× wash buffer (KPL, Inc.; Gaithersburg, MD) using a standard wash protocol on an AquaMax 200 plate washer (Molecular Devices, LLC; Sunnyvale, CA). The plate was then blocked with 1× milk diluent block (MDB; KPL, Inc.) for 30 min at 37 °C. The plate was washed, and 100 µL of PBS with 0.05% Tween 20 (PBS-T; Sigma-Aldrich) was applied to each well in the plate. MS2CP was diluted in PBS-T to 2 µg/mL, and 100 µL of this solution was applied to the first well of each row. A 2-fold serial dilution was performed across the plate, and then it was incubated for 1 h at 37 °C.

After the plate was washed, rabbit anti-MS2 (supplied by JPMO BSV CRP) was diluted to 5 µg/mL in 1× MDB, and 100 µL of this solution was added to each well. The plate was then incubated at 37 °C for 1 h. After incubation, the plate was washed. Goat anti-rabbit IgG (H+L)[*]-horseradish peroxidase (HRP; KPL, Inc.) was diluted to 0.2 µg/mL in 1× MDB, and 100 µL of this solution was added to each well. The plate was incubated at 37 °C for 30 min. After the plate was washed, 100 µL of room-temperature 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) one-component HRP substrate (KPL, Inc.) was added to each well. After 20 min at 37 °C, the optical density at the 405 nm light wavelength was determined using a Synergy H4 hybrid multi-mode microplate reader (BioTek Instruments, Inc.; Winooski, VT). Data analysis was performed using Prism software (GraphPad Prism, version 5.00 for Windows, GraphPad Software; San Diego, CA).

2.8 SPR Methodology

SPR is one method used to determine the kinetic parameters of antibody–antigen interactions. It is a rapid methodology for monitoring biomolecular interactions through excitation of surface plasmons. Polarized light is shone through a prism on a sensor chip with a thin metal film coating, which reflects the light by acting as a mirror. If the angle of light shone through the prism is changed, and the intensity of the reflected light is monitored, differences in intensity can be recorded. Although the refractive index at the prism side of the sensor chip does not change, the refractive index in the immediate vicinity of the metal surface will change when

[*] H+L, heavy and light chains of the antibody.

accumulated mass (bound proteins) adsorbs on the surface. Therefore, if binding occurs, the resonance angle (SPR angle) changes, and this SPR angle shift provides information on the protein adsorption kinetics on the surface. The software can then be used to provide an accurate analysis of the association (k_a) and dissociation (k_d) rate constants for the antibody interactions and to calculate the overall affinity constant (K_D) between antibody and antigen.

2.8.1 Thermostability Testing Using SPR

Using a Biacore T200 system (GE Healthcare), 6500 response units (RUs) of MS2CP was tethered to one flow cell of a Biacore CM5 sensor chip using standard amine coupling chemistry. After a thermal stress test was performed, samples were centrifuged at 2000 × *g* and 5 °C for 5 min. The analyte was run at 10 µL/min for 120 s. A calibration curve was created by injecting eight concentrations of the time 0 unheated, NRL MS2 sdAb samples (2cams2 or B1-AP) at 400, 350, 300, 250, 200, 150, 100, and 50 nM and plotting their respective analyte-binding capacities of the surfaces in RU (R_{Max}). Unheated and heated samples were then diluted 1:90 and 1:180 so that the time 0 control points fall on the linear calibration curve. All samples were run in triplicate. The chip's surface was regenerated with an 18 s injection of 0.85% phosphoric acid at a flow rate of 30 µL/min. Data was collected using the Biacore concentration analysis software, and the active concentration of heated sample was recorded. The running buffer used for this experiment was Biacore HBS-EP 1× buffer (GE Healthcare Life Sciences).

2.8.2 Kinetic Analysis Using SPR

Using a Proteon XPR36 SPR system (Bio-Rad) and PBS-T running buffer, 200 RU of MS2CP was tethered to a GLC sensor chip (Bio-Rad) using standard amine-coupling chemistry. NRL MS2 sdAb (2cams2 or B1-AP) was injected across the chip's surface for 120 s at a flow rate of 100 µL/min with 600 s of dissociation at 5 nM, 1.67 nM, 560 pM, 190 pM, and 60 pM. The chip's surface was regenerated using an 18 s injection of 0.85% phosphoric acid at 100 µL/min. Data was analyzed using a Langmuir 1:1 fit.

3. RESULTS

3.1 Spectrophotometry Results

Both of the NRL MS2 sdAbs were read in triplicate on the NanoDrop ND-1000 spectrophotometer. The A_{280} readings are shown in Table 1.

Table 1. NanoDrop A_{280} Readings

Replicate	NRL MS2 2cams2 (mg/mL)	NRL MS2 B1-AP (mg/mL)
1	2.34	4.14
2	2.32	4.13
3	2.31	4.10

For each antibody, these three readings (Table 1) were averaged and divided by the extinction coefficient of 1.44. The final concentrations were determined to be 2.32 mg/mL for the thermally stabilized NRL MS2 2cams2 and 4.12 mg/mL for affinity-matured NRL MS2 B1-AP.

3.2 Electrophoresis Results

The molecular weights of the NRL MS2 sdAbs were determined using the Experion Pro260 analysis kit, as shown in Figure 1. The thick bands observed in the second and fourth lanes correspond to the MS2 sdAbs 2cams2 and B1-AP, respectively. According to the results of the Experion software analysis, 2cams2 was only 49.9% pure and weighed 19.0 kDa, and B1-AP was 98.1% pure with a heavy chain of 49.5 kDa and a light chain of 19.5 kDa.

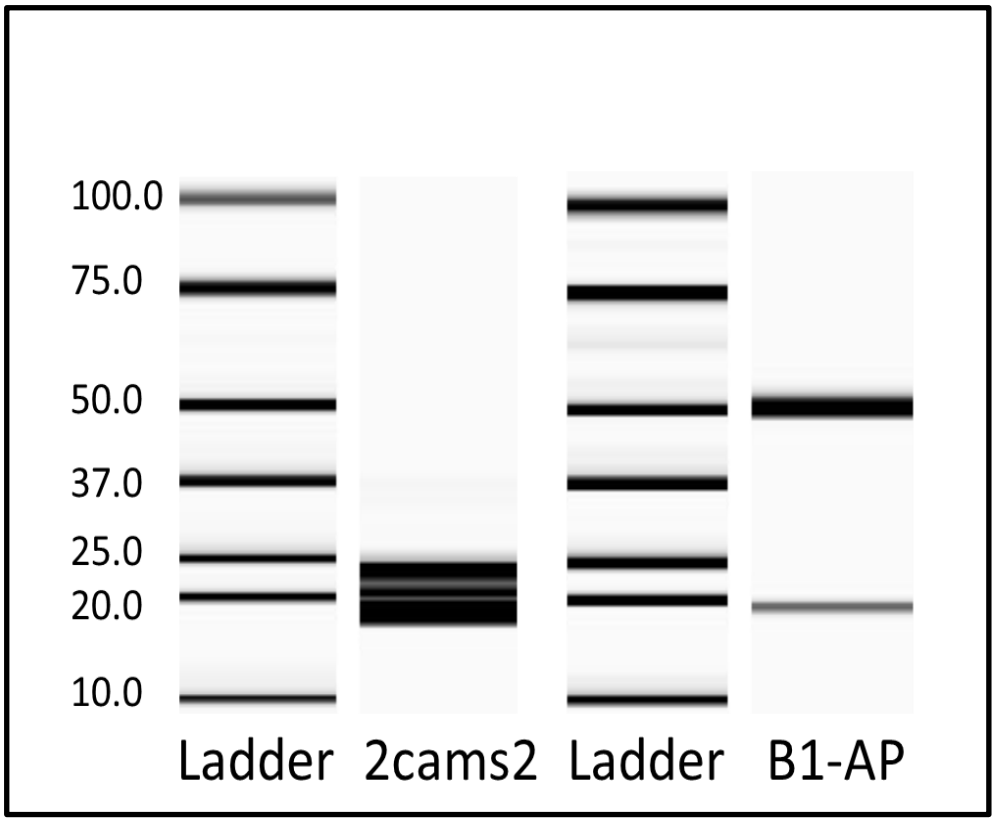


Figure 1. Molecular weight and purity of the two NRL MS2 sdAbs. Digital gel of NRL MS2 sdAbs produced by the Experion Pro260 analysis kit. The thick band shown in the second lane corresponds to the NRL MS2 sdAb 2cams2, and the bands in the fourth lane correspond to the heavy and light chains of NRL MS2 sdAb B1-AP.

3.3 DLS Results

Both of the NRL MS2 sdAbs were analyzed in triplicate using the DynaPro plate reader. The radius of NRL MS2 2cams2 was determined to be 2.3 nm, with a polydispersity of 15.9% (Table 2), whereas the radius of NRL MS2 B1-AP was determined to be 3.6 nm with a polydispersity of 10.4%. Figure 2 contains representative correlation and regularization graphs for each of the NRL MS2 sdAbs. The correlation graphs (Figures 2A and 3A) depict a sigmoidal curve, which is indicative of a valid size distribution. The regularization graphs (Figures 2B and 3B) illustrate the monodispersity found in both samples. Table 2 shows the raw data produced for each replicate. Because 100% of the mass displays favorable polydispersity and hydrodynamic radius, both of these sample preparations are considered to be monodisperse.

Table 2. Features of MS2 sdAbs in Solution

Sample	Replicate	Radius (nm)	Polydispersity (%)
NRL MS2 2cams2	1	2.5	24.9
	2	2.4	21.3
	3	2.2	9.7
	4	2.1	11.7
	5	2.1	11.8
	Average	2.3	15.9
NRL MS2 B1-AP	1	3.5	11.1
	2	3.8	13.7
	3	3.6	8.8
	4	3.6	9.9
	5	3.6	8.4
	Average	3.6	10.4

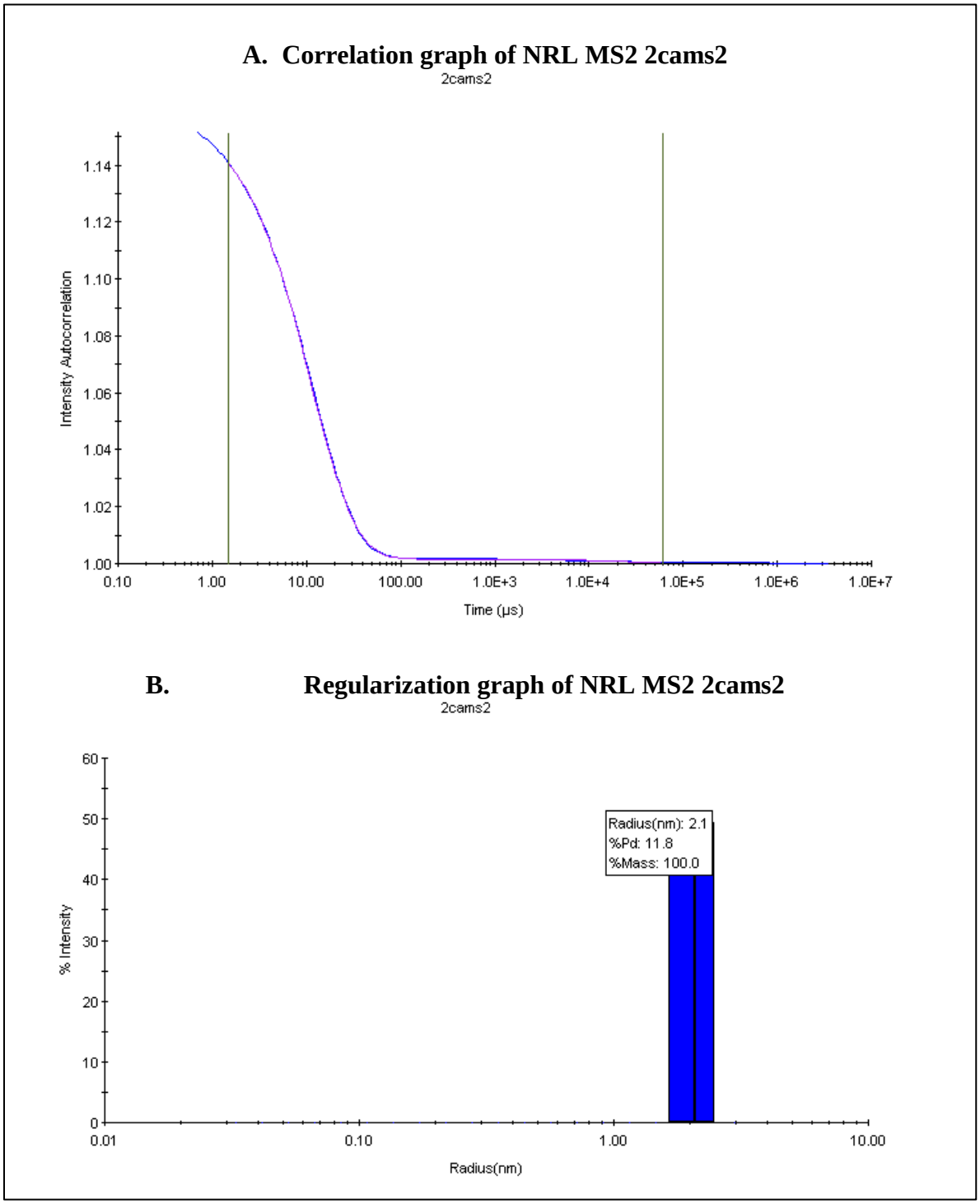


Figure 2. Radius and polydispersity representations of thermostable NRL MS2 sdAb 2cams2. (A) Correlation and (B) regularization graphs of the NRL thermostable sdAb 2cams2.

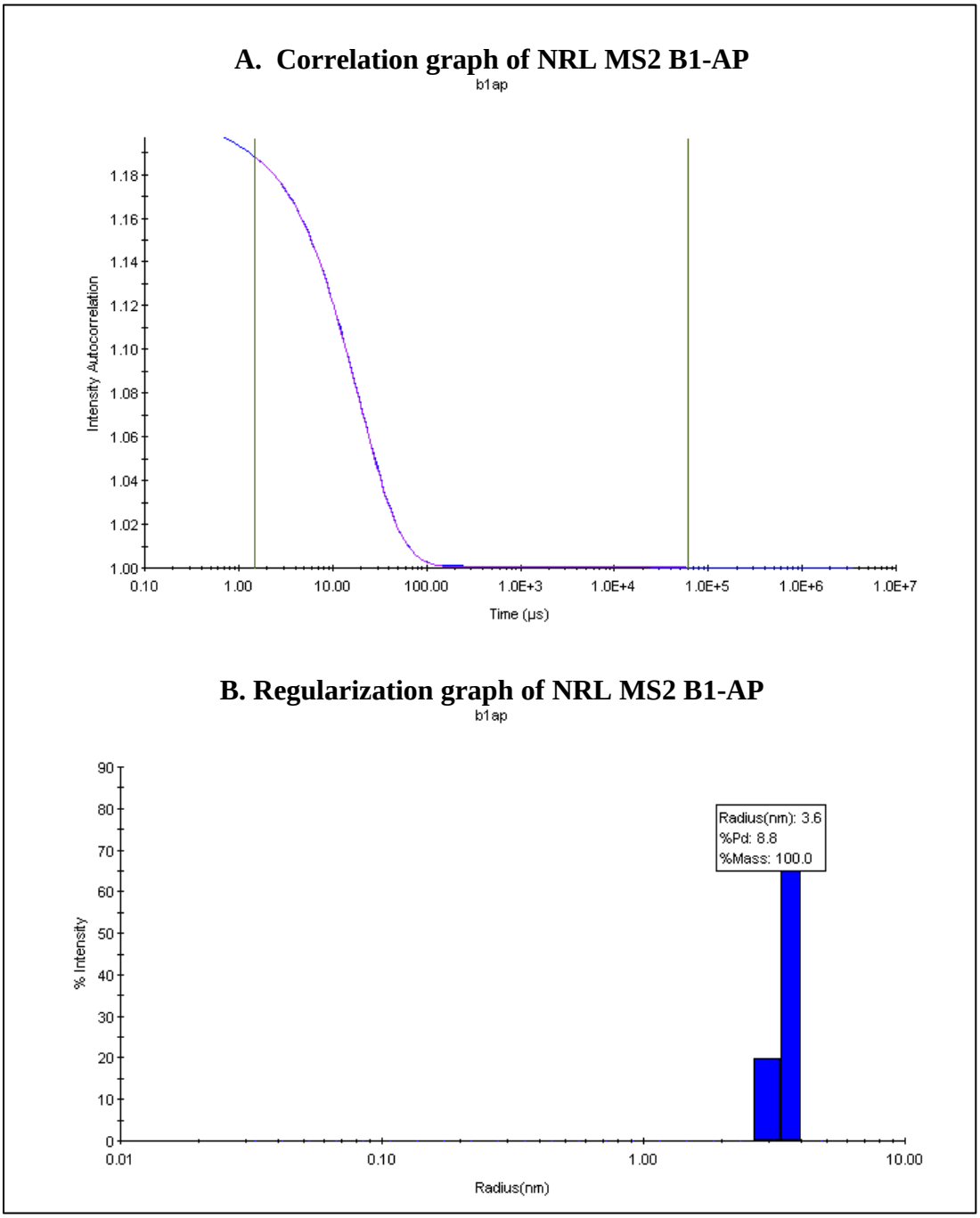


Figure 3. Radius and polydispersity representations of affinity-matured NRL MS2 sdAb B1-AP. (A) Correlation and (B) regularization graphs of the NRL affinity-matured sdAb B1-AP used for determining the radius and polydispersity of the samples.

3.4 DSC Results

The NRL MS2 sdAbs were analyzed in duplicate using the MicroCal VP-DSC instrument. The peak T_m values were as follows:

- NRL MS2 2cams2 Replicate 1: 67.2 °C;
- NRL MS2 2cams2 Replicate 2: 62.2 °C;
- NRL MS2 B1-AP Replicate 1: 67.5 °C; and
- NRL MS2 B1-AP Replicate 2: 67.5 °C.

The final T_m for NRL MS2 2cams2 was determined to be 64.7 °C (Figure 4), and that of NRL MS2 B1-AP was determined to be 67.5 °C (Figure 5).

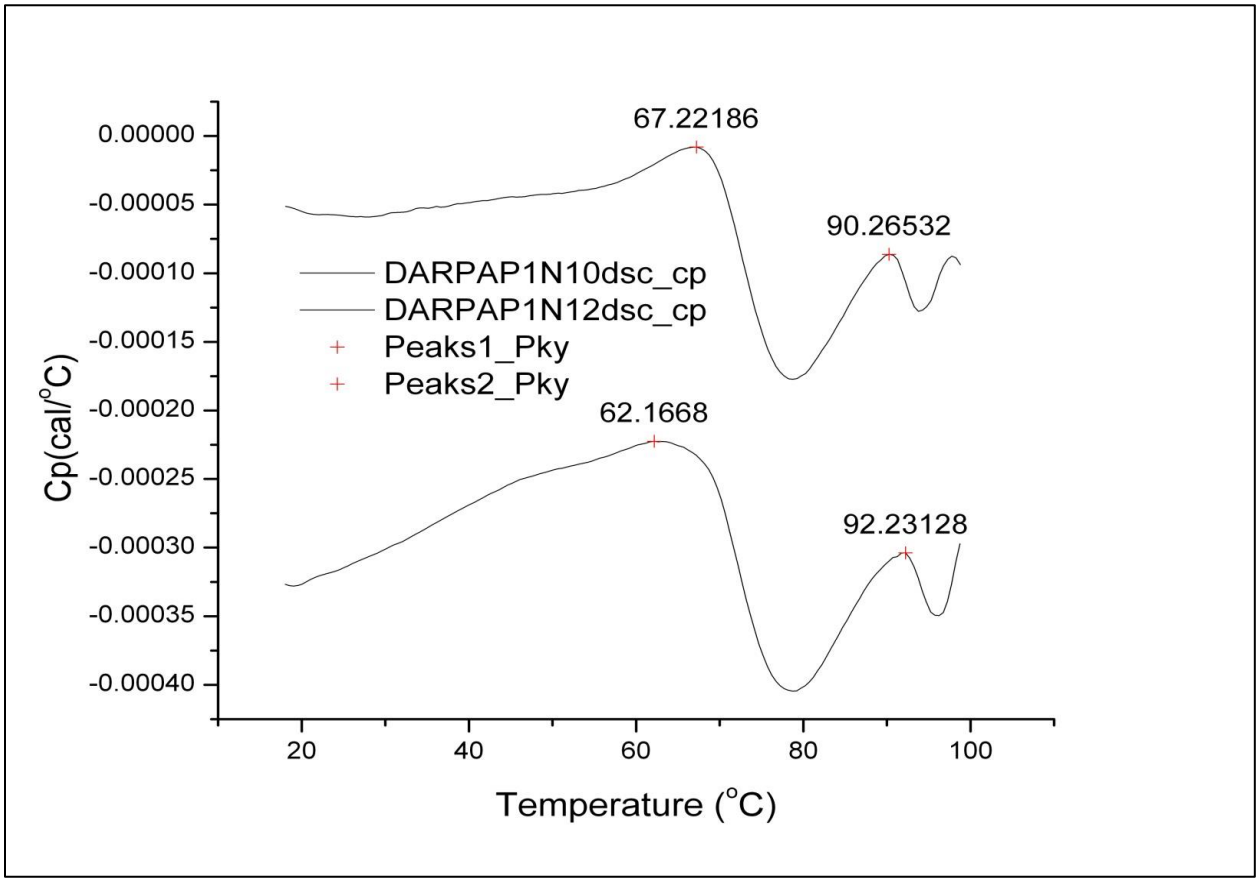


Figure 4. Transition midpoint curve for NRL MS2 2cams2. Curves were generated on the MicroCal VP-DSC microcalorimeter and analyzed using the Peak Find function in the Origin 7.0 software. The transition midpoint was calculated to be 64.7 °C for NRL MS2 2cams2.

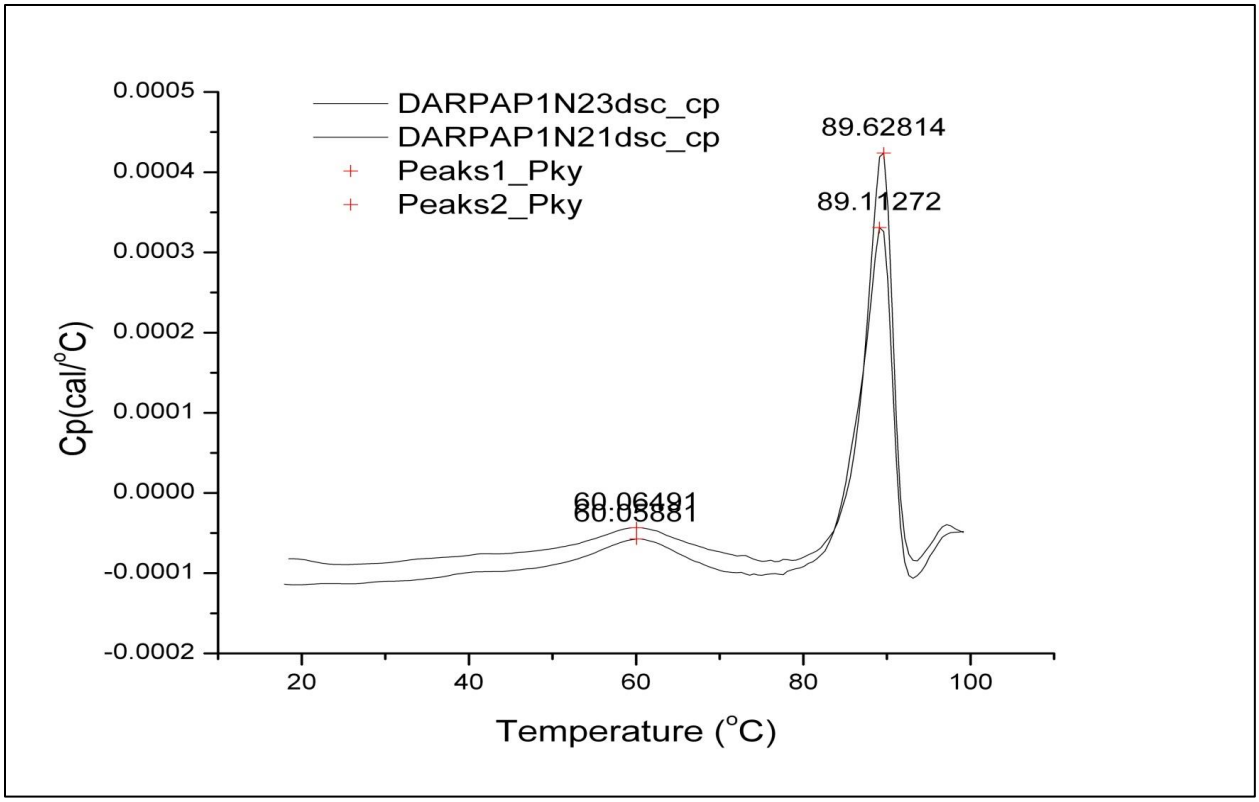


Figure 5. Transition midpoint curve for NRL MS2 B1-AP. Curves were generated on the MicroCal VP-DSC microcalorimeter and analyzed using the Peak Find function in the Origin 7.0 software. The transition midpoint was calculated to be 67.5 °C for NRL MS2 B1-AP. The other peak represents the molecule added for stability.

3.5 ELISA Results

ELISAs were used to test the functional interactions of antibodies and antigens after thermal stress at 70 ºC. The ELISA data (Figure 6) show that when the NRL sdAb 2cams2 was heated to 70 °C, it maintained activity during 45 min of thermal stress, unlike the government-supplied MS2 scFv, which lost activity after 15 min and did not maintain activity over the full hour of thermal stress. The curves in Figure 6A show antibody activity for different time points at 70 °C, as a function of the concentration of antigen supplied. The area under the curve for each of the different time points at 70 °C was calculated, averaged, and graphed (Figure 6B) to show how the MS2 sdAb reacted to thermal stress over time. Evaluation of this graph indicates that some of the MS2 sdAb remains functionally capable of binding to antigen after a 45 min exposure to 70 °C, unlike the original MS2 scFv, which lost activity within 15 min of heating.

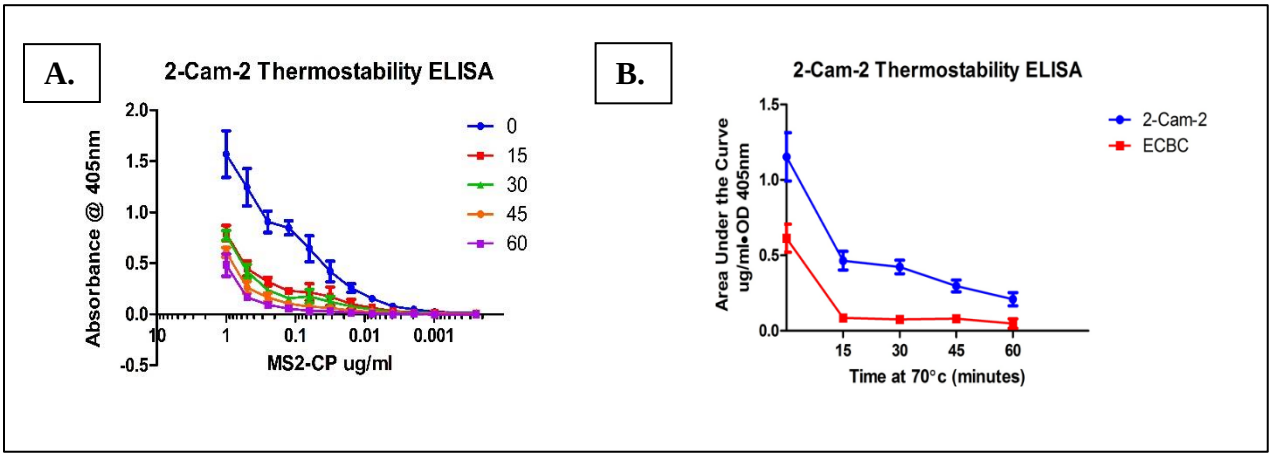


Figure 6. Thermostability of NRL MS2 2cams2 using ELISA. (A) Activity of NRL MS2 2cams2 as a function of antigen concentration after thermal stress. The different curves are represented on the legend by the number of minutes the sample was held at 70 °C. (B) Area under the curve analysis depicting the effect of thermal stress.

3.6 SPR Results

3.6.1 Thermostability Testing Results Using SPR

After heating the antibody–antigen complex to 70 °C for variable time periods, SPR was used to assess the functional binding between the NRL MS2 sdAb (2cams2) and antigen. Five tubes of 1 mg/mL MS2 sdAb were prepared and heated to 70 °C for the following time periods: 15, 30, 45, and 60 min, followed by quenching the tubes on ice. A Biacore T200 system was used to compare the activity of each sample with a calibration curve for unheated samples. The percent activities of the heated samples were plotted over time (Figure 7). The results indicated that 2cams2 did not maintain over 85% activity over the full 60 min of thermal stress, but it did remain active over the first 45 min, whereas the activity of the scFv dropped off completely within the first 15 min at 70 °C. However, this amount of activity did not meet the thermostability requirement.

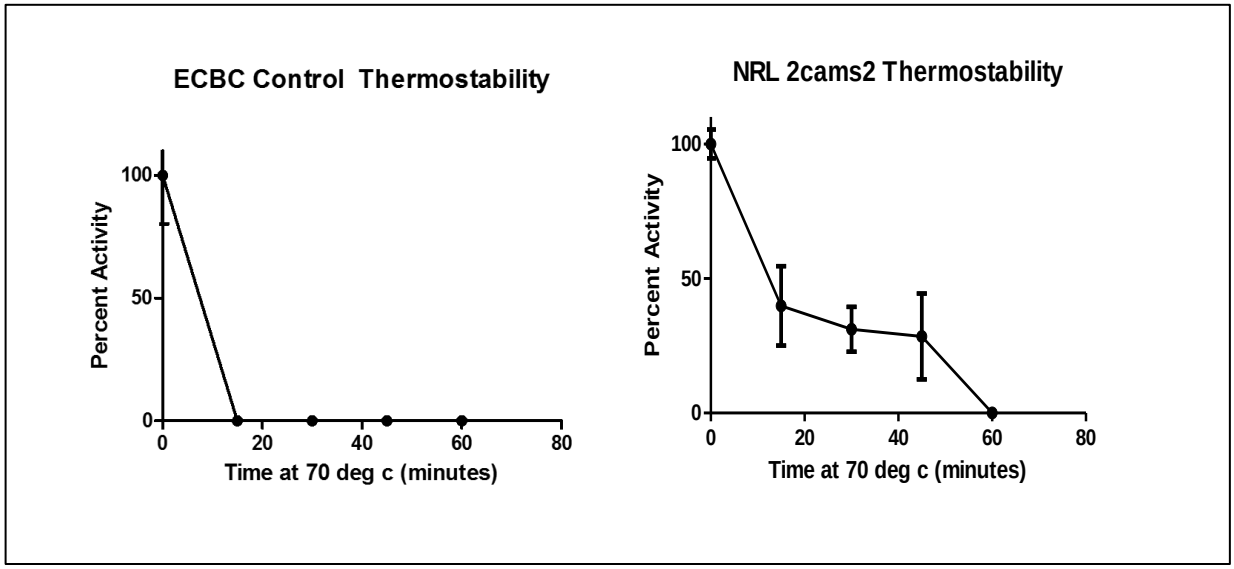
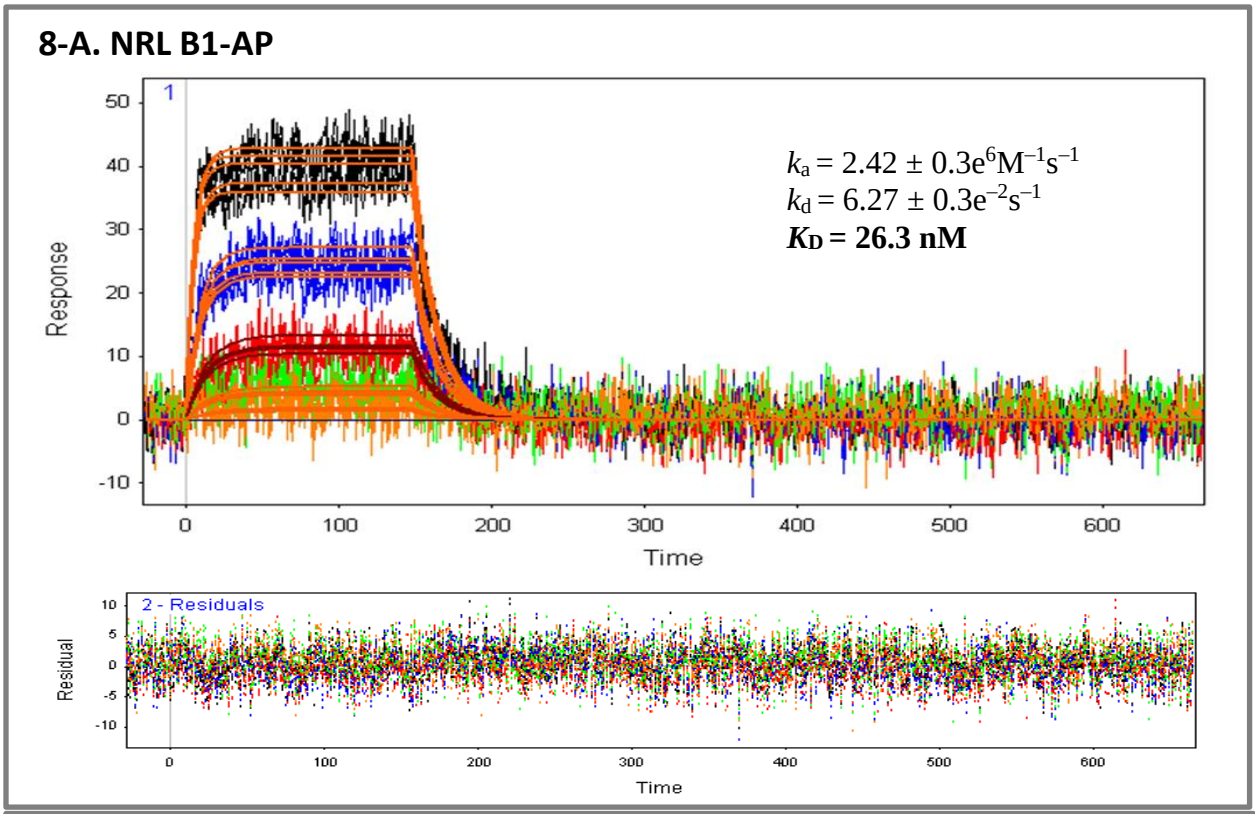


Figure 7. Thermostability of the NRL MS2 sdAb 2cams2 compared with the ECBC MS2 scFv obtained using SPR. The NRL antibody maintained activity after heating to 70 ºC for 45 min, compared with the original ECBC MS2 scFv antibody, which lost all ability to recognize the MS2CP target within 15 min of heating.

3.6.2 Kinetic Analysis Results Using SPR

Kinetic analysis of the affinity-enhanced NRL MS2 sdAb B1-AP binding to the MS2CP antigen was performed as a direct-binding SPR experiment on the Proteon XPR36 system, and the results are presented in Figure 8A. Data was normalized to a blank-immobilized reference flow cell and fit to a Langmuir 1:1 model using the Bio-Rad Proteon XPR36 software. The K_D was determined to be 26.3 nM. Results from similar experiments run using the original ECBC MS2 scFv are presented in Figure 8B. The K_D of the original scFv was determined to be 8.66 nM; therefore, the NRL staff provided an antibody that did not meet the 100-fold improvement threshold. Finally, kinetics analysis was also performed on the thermostable-enhanced NRL MS2 sdAb 2cams2 and the results are presented in Figure 8C. The K_D was determined to be 22.6 nM.



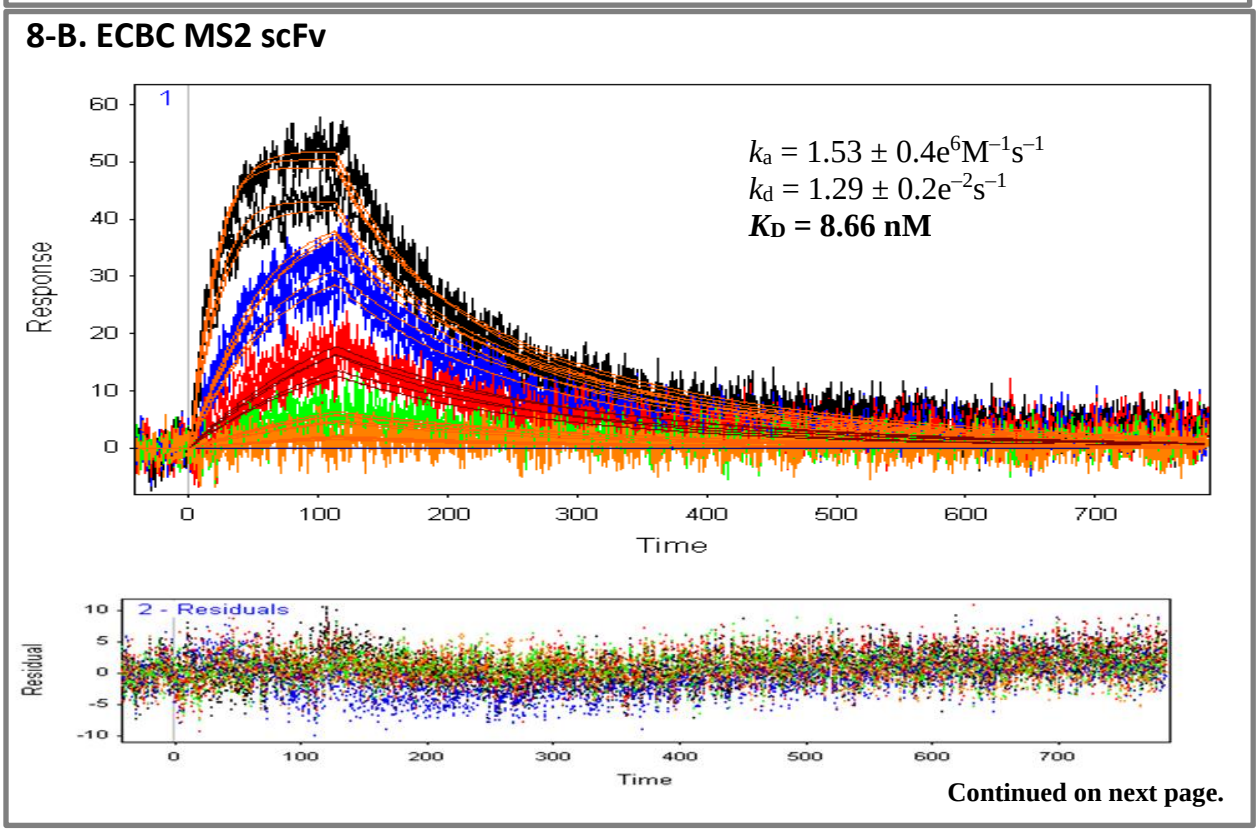


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Figure 8. Comparison of the kinetic fits with residuals of the MS2 antibodies, determined using a Proteon XPR36 system. (A) Kinetics of NRL affinity-enhanced MS2 sdAb B1-AP (26.3 nM) and (B) kinetics of original MS2 scFv (15.5 nM).

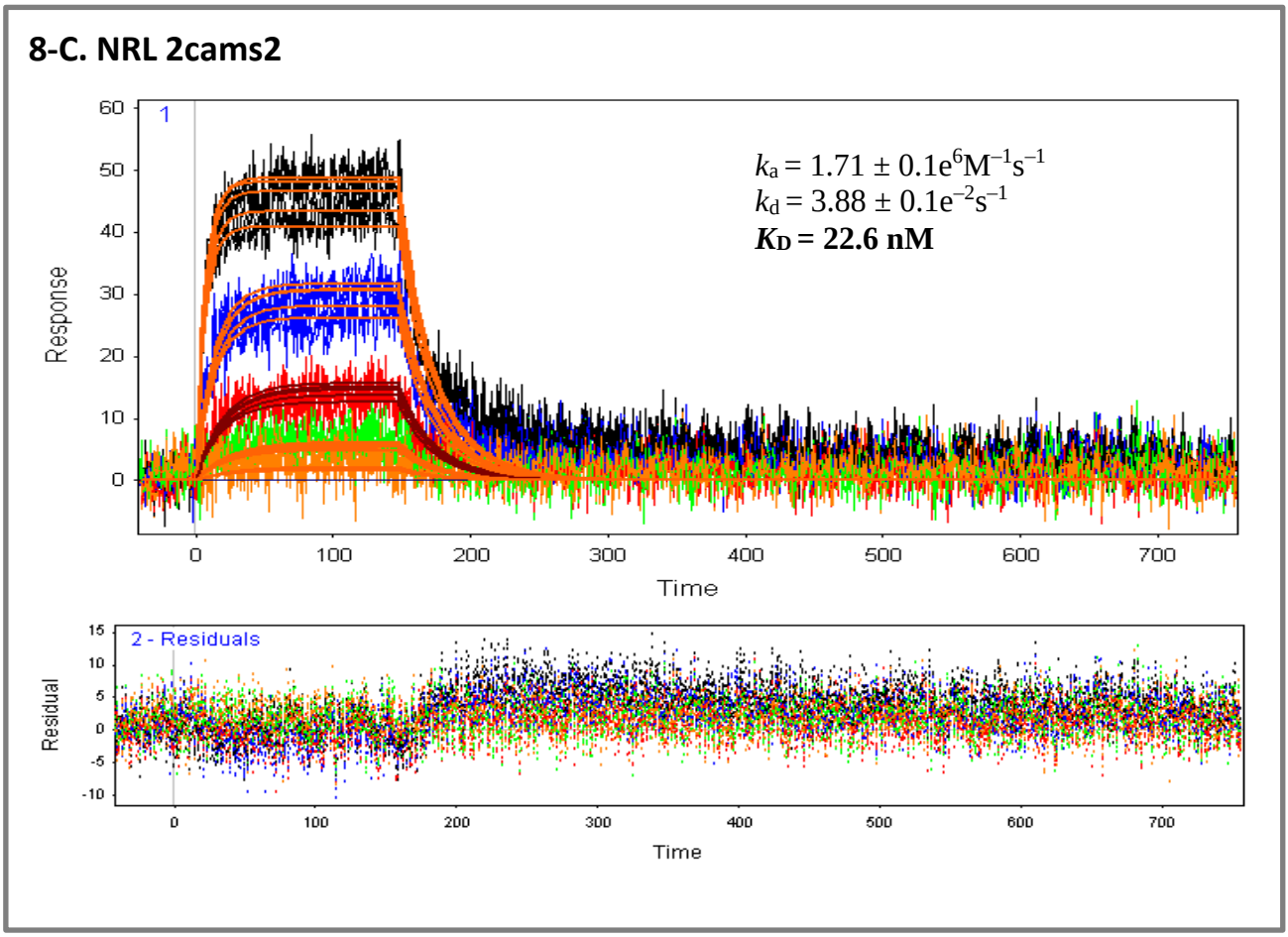

Figure 8 (continued). Comparison of the kinetic fits with residuals of the MS2 antibodies, determined using a Proteon XPR36 system. (C) Kinetics of NRL thermostable-enhanced MS2 sdAb 2cams2 (22.6 nM).

4. DISCUSSION

This study established and standardized the parametric tests for performance on the MS2 scFv antibody, which was selected by the DARPA ATP as the initial substrate to be used to demonstrate molecular schemes for improving the thermal stability and affinity of an antibody for its target antigen. The test bed that was developed for this study was used to define the physical and functional properties of the reference MS2 scFv antibody and establish the baseline for subsequent testing of the engineered antibodies submitted by the ATP performers. A snapshot of the MS2 scFv physical characteristics was obtained using the NanoDrop, Experion, and DLS measurement platforms. Measurements of the MS2 scFv functional characteristics, used for assessing the effects of molecular engineering on thermal stability and affinity, were obtained using the DSC, ELISA, and SPR analytic platforms.

An accurate assessment of protein concentration is critically important for all of the test procedures described in this report. We applied the standard technique of

spectrophotometry with the NanoDrop ND-1000 system. This instrument was employed to provide the A_{280} value of the sample, which is influenced by the number of tryptophan and tyrosine residues in a given protein. For this reason, the extinction coefficient was used in conjunction with A_{280} value to determine an accurate concentration.

After concentration was determined using spectrophotometry with the NanoDrop system, molecular weight and purity data were collected with the Experion automated electrophoresis system, which employs microfluidic technology to automate electrophoresis for protein analysis. The results of the Experion system analysis of the MS2 sdAb protein fell within the acceptable range of purity for use in assay development, and the molecular weight determined by the software (shown in Figure 1) was typical for an sdAb.

DLS was used in conjunction with the Experion and NanoDrop systems to illustrate how the protein behaved in solution. The DLS data indicate the physical state and potential aggregation of a protein in solution by measuring the polydispersity, hydrodynamic radius, and molecular weight of a sample. The DLS data were used to establish whether the MS2 sdAbs provided by NRL were monomeric and monodisperse. Less than 1% of the sample mass appeared to be aggregating in solution (Figures 2 and 3). To mitigate the exacerbating effect of freeze-thawing on future sample aggregation and provide consistent testing, the NRL MS2 sdAbs were aliquoted into single-use vials and centrifuged before use.

In the next round of testing, the thermostabilities of the NRL MS2 sdAbs were evaluated using DSC, ELISA, and SPR. DSC was used to obtain a quantitative T_m for the MS2 sdAbs. The T_m can be used to predict the results of ELISA and SPR thermostability testing. A T_m above 70 °C predicts that the percent activity of the MS2 sdAbs after thermal stress should remain above 50%. A T_m below 70 °C predicts at least a 50% decrease in MS2 sdAb activity after thermal stress. The NRL MS2 sdAb 2cams2, which was optimized for thermostability and described herein, exhibited a T_m of 64.7 °C (Figure 4). This was compared with a T_m from the original MS2 scFv of 67.5 °C (*4*). Therefore, it was expected that heating the NRL MS2 sdAb 2cams2 above 70 °C would cause the sample to unfold and lose at least 50% of its activity when evaluated using ELISA and SPR.

The results of the thermal stress test demonstrated that the NRL MS2 sdAb did not remain active for over 60 min of heating at 70 °C. Evaluation of the ELISA and SPR data confirmed that the NRL sdAb 2cams2 was able to bind the MS2CP after 45 min of heating but not for the full 60 min (Figures 6 and 7). This result was unlike that of the original MS2 scFv reference antibody, which was unable to bind the MS2CP after only 15 min of heating at 70 °C.

SPR was also used to obtain a kinetic analysis of the affinity-enhanced NRL MS2 sdAb B1-AP binding to its target antigen (MS2CP) to compare binding parameters with the original antibody. Kinetic data for the binding of B1-AP MS2 sdAb to the MS2CP was obtained using the Proteon XPR36 SPR platform, which yielded a K_D of 26.3 nM, and the original MS2 scFv yielded a K_D of 8.66 nM (Figure 8). The K_Ds clearly show that the NRL results did not meet the 100-fold improvement requested by DARPA.

5. CONCLUSION

The DARPA ATP sought to establish methods for rapidly engineering a given antibody reagent that would exhibit physical and functional properties far exceeding those of its native state, thereby expanding user confidence in fielding antibody-based detection and diagnostic platforms for use in environments or operational scenarios that would degrade or interfere with the currently available reagents. By optimizing the thermal stability and binding affinity of an antibody for its biological target, the DARPA ATP will develop antibody reagents that can reliably function in harsh environmental conditions and increase the sensitivity of sensor platforms to detect lower levels of a threat agent.

This report documents the testing of an improved, thermostable antibody (2cams2) and an affinity-improved antibody (B1-AP), which were both produced by NRL. This study evaluated the physical and functional characteristics of these sdAbs in the ECBC testing pipeline. The results were compared to the baseline characteristics of the original antibody's physical properties to include concentration, molecular weight, purity, state of aggregation in solution, and functional measures such as binding affinity and thermal stability. Neither NRL antibody exhibited enhanced thermal stability or affinity for binding to the MS2CP antigen.

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ACRONYMS AND ABBREVIATIONS

A_{280}	absorbance of light at 280 nm
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ATP	Antibody Technology Program
BGG	bovine γ-globulin
CRP	Critical Reagents Program
DARPA	Defense Advanced Research Projects Agency
DLS	dynamic light scattering
DSC	differential scanning calorimetry
ECBC	U.S. Army Edgewood Chemical Biological Center
ELISA	enzyme-linked immunosorbent assay
HRP	horseradish peroxidase
JPMO BSV	Joint Product Management Office for Biosurveillance
k_a	association rate constant
k_d	dissociation rate constant
K_D	affinity constant
MDB	milk diluent block
MS2CP	MS2 coat protein
NRL	U.S. Naval Research Laboratory
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline with 0.05% Tween 20
R_{Max}	maximum analyte-binding capacity of the surface in RU
RU	response unit
scFv	single-chain fragment variable
sdAb	single domain antibody
SPR	surface plasmon resonance
T_m	melting temperature
UV-Vis	ultraviolet-visible spectroscopy

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